Duplex Real-Time PCR Assay for Detection of *Streptococcus pneumoniae* in Clinical Samples and Determination of Penicillin Susceptibility[▽]

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We have developed a duplex real-time PCR for the rapid diagnosis of *Streptococcus pneumoniae* infection from culture-negative clinical samples with the simultaneous determination of penicillin susceptibility. The assay amplifies a *lytA* gene target and a penicillin binding protein 2b (*pbp2b*) gene target in penicillin-susceptible organisms. The assay was shown to be sensitive (detects 0.5 CFU per PCR) and specific for the detection of *S. pneumoniae* DNA. The assay was validated by comparing *pbp2b* PCR results with MIC data for 27 *S. pneumoniae* isolates. All 5 isolates with penicillin MICs of >1.0 mg/liter were *pbp2b* real-time PCR negative, as were 9 of the 10 isolates with penicillin MICs of 0.12 to 1.0 mg/liter. One isolate with a penicillin MIC of 0.12 to 1.0 mg/liter gave an equivocal *pbp2b* real-time PCR result. Twelve isolates were penicillin susceptible (MICs of ≤0.06 mg/liter) and *pbp2b* real-time PCR positive. These data were used to establish an algorithm for the interpretation of penicillin susceptibility from the duplex PCR result. *pbp2b* real-time PCR results were also compared to an established PCR-restriction fragment length polymorphism (RFLP) method previously applied to these 27 isolates and 46 culture-negative clinical samples (containing *S. pneumoniae* DNA by broad-range 16S rRNA gene PCR). Discordant results were seen for four isolates and six culture-negative clinical samples, as PCR-RFLP could not reliably detect penicillin MICs of 0.12 to 1.0 mg/liter. We report prospective application of the duplex PCR assay to the diagnosis of *S. pneumoniae* infection from 200 culture-negative clinical specimens sent to the laboratory for diagnostic broad-range 16S rRNA gene PCR. One hundred six were negative in the duplex PCR. Ninety-four were *lytA* PCR positive, and 70 of these were also *pbp2b* PCR positive and interpreted as penicillin susceptible. Fourteen were *pbp2b* PCR negative and interpreted as having reduced susceptibility to penicillin. For the remaining 10 samples, susceptibility to penicillin was not determined.

Culture of clinical specimens for *Streptococcus pneumoniae* is slow and is often rendered negative by antibiotic treatment prior to sampling. PCR has been shown to be a useful tool for the rapid identification of *S. pneumoniae* from both clinical samples and bacterial isolates. Various PCR methods have been used to identify *S. pneumoniae*, including amplification of the genes coding for two pneumococcal virulence factors, autolysin (*lytA*) (4, 10) and pneumolysin (*ply*) (7, 18). However, there are reports that *lytA* and *ply* PCR may not be specific for the detection of *S. pneumoniae*, as some streptococcal species in the *S. mitis* group (SMG) may also harbor these genes. Such SMG strains appear to be associated with severe disease. Recent studies have found that *lytA* PCR was more specific than *ply* PCR for the identification of *S. pneumoniae* (11, 20, 21).

Penicillin has been the cornerstone of treatment for pneumococcal infections; however, the emergence and spread of reduced susceptibility to penicillin has been noted throughout the world (16). Figures for the year 2000 revealed that 7% of isolates from cases of invasive pneumococcal disease in the United Kingdom had reduced sensitivity to penicillin (3). The definition of MIC breakpoints for pneumococcal isolates to penicillin has been the subject of debate and frequent modification. Currently the Clinical and Laboratory Standards Institute (CLSI) (http://www.clsi.org) and the British Society for Antimicrobial Chemotherapy (BSAC) (http://www.bsac.org.uk) both define strains with penicillin MICs of ≤0.06 mg/liter as susceptible and those with penicillin MICs of >1.0 mg/liter as resistant. Interpretation of strains with penicillin MICs of 0.12 to 1.0 mg/liter varies according to the site of infection, the route of drug administration, and which advisory body's guidelines are used. Reduced susceptibility to penicillin and other β-lactam antibiotics may be mediated by the production of altered penicillin binding proteins (PBP-1A, -1B, -2A, -2B, -2X, and -3) which have reduced affinities for penicillin. Production of altered PBP-2B as a result of sequence polymorphisms in the *pbp2b* gene appears to be particularly important (19) and may result in treatment failure if penicillin is used. Agents useful for treatment of penicillin-resistant pneumococcal infections include the expanded-spectrum cephalosporins, rifampin, linezolid, the glycopeptides, and the newer fluoroquinolones (9). All such agents are of a broader spectrum and potentially more toxic or more expensive than penicillin and ideally should be reserved for the treatment of infection with penicillin-resistant organisms.

The increased use of molecular tests such as PCR for the diagnosis of bacterial infection has led in turn to an increased demand for antibiotic susceptibility testing using molecular

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TABLE 1. Primer and probe information

Primer/probe	Source or reference	Sequence[a]	Target gene (position)[b]
Pbp-2b-JT-F	This study	5′-CTC AGG TGG CTG TTA TTG C-3′	pbp2b (614–632)
Pbp-2b-JT-R	This study	5′-GCG CAA TGG AAG GTC CTA C-3′	pbp2b (1996–2014)
LytA-F	10	5′-ACG CAA TCT AGC AGA TGA AGC-3′	lytA (327–347)
LytA-R	10	5′-TGT TTG GTT GGT TAT TCG TGC-3′	lytA (407–427)
LytA-probe	10	FAM 5′-TTT GCC GAA AAC GCT TGA TAC AGG G-3′ TAMRA	lytA (351–375)
Pbp-2b-F	8	5′-ATT CTT GGT ATA CTC AGG CT-3′	pbp2b (1280–1299)
Pbp-2b -RMOD	This study	5′-TGT TTG GAC CAT ATA GGT ATT-3′	pbp2b (1348–1368)
Pbp-2b-R	8	5′-GTT TGG ACC ATA TAG GTA TTT-3′	pbp2b (1349–1369)
Pbp-2b-probe	8	VIC 5′-CAC AGC GGT CCA AGC TCT-3′ TAMRA	pbp2b (1317–1334)
F2	This study	5′-TGT TCC AGG TTC GGT TGT-3′	pbp2b (1161–1178)
R4	This study	5′-GAC TCT AGA TTG CTG GTG C-3′	pbp2b (1415–1433)

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.
[b] S. pneumoniae R6 (GenBank no. AE008520).

methods. However, unlike phenotypic testing for antibiotic susceptibility, which examines all resistance mechanisms for a particular antibiotic simultaneously, molecular testing can detect only known resistance mechanisms. To date, the only penicillin resistance mechanism described for *S. pneumoniae* that relates to treatment failure is an altered *pbp2b* gene. Several molecular methods for detecting mutations in this gene that predict MIC have been published, including restriction fragment length polymorphism (RFLP) analysis of *pbp2b* PCR products. Penicillin-susceptible strains were shown to have a characteristic RFLP band pattern when the amplified *pbp2b* gene was digested with HinfI. Strains with reduced susceptibility gave rise to a number of different patterns (2, 15).

More recently, a real-time PCR assay for the detection of penicillin susceptibility of *S. pneumoniae* strains has been described (8). The assay amplifies a conserved region of the *pbp2b* gene in penicillin-susceptible "wild-type" *S. pneumoniae*; failure to detect a product infers reduced penicillin susceptibility (MICs of >0.06 mg/liter) (5, 8). A real-time *pbp2b* PCR could provide information on penicillin susceptibility more rapidly than our current PCR-RFLP assay and could feasibly provide prospective clinically applicable data on penicillin susceptibility from culture-negative samples positive for *S. pneumoniae* DNA by PCR.

Previous studies at our center found that *S. pneumoniae* was the most frequent organism detected by broad-range 16S rRNA gene PCR in culture-negative specimens (6) and in particular in culture-negative empyema fluid (17). However, even after broad-range 16S rRNA gene PCR, one-third of culture-negative empyema fluids remain negative. A specific *S. pneumoniae* real-time PCR that is faster and more sensitive than the broad-range PCR could result in improved detection of microbial DNA in culture-negative samples and, when combined with reliable detection of penicillin susceptibility, could drive the rational prescription of narrow-spectrum antibiotics. The purpose of this study was to develop and validate a duplex real-time PCR for the rapid detection of *S. pneumoniae* in culture-negative clinical specimens with simultaneous demonstration of penicillin susceptibility (MICs of ≤0.06 mg/liter).

MATERIALS AND METHODS

Samples. The specimen types tested included blood, cerebrospinal fluid, empyema fluid, pleural fluid, other respiratory samples, pericardial fluid, pus, and tissue. Extracted DNA samples from 246 clinical specimens submitted for broad-range 16S rRNA gene PCR were tested. Forty-six of these were archived DNA extracts (stored at −70°C) used for the validation of the assay. The remaining 200 samples were sent for 16S rRNA gene PCR between January 2006 and August 2007 and were tested prospectively in the assay. Details of the extraction procedure have been described previously (6).

Isolates. A total of 35 streptococcal isolates were examined. Twenty-three of these were isolated in our laboratory, 15 were identified as *S. pneumoniae*, and 8 were identified as SMG by phenotypic methods, by optochin sensitivity, and by use of API Strep (Biomerieux UK Ltd., Basingstoke, United Kingdom). All eight SMG isolates were considered to be clinically significant; five were isolated from blood cultures, one was isolated from a cerebrospinal fluid sample, and one was isolated from a nasal pharyngeal aspirate. All isolates were eventually identified as SMG, and some *S. pneumoniae* isolates required 16S rRNA gene sequencing to confirm their identity (6). Twenty-two isolates were from clinical material and 1 (a penicillin-resistant *S. pneumoniae* isolate) was from a United Kingdom National External Quality Assessment Service (UK NEQAS) sample. DNA was extracted using a previously published method (6). MICs to penicillin for these 23 isolates were determined in our laboratory by the E-test method (Biostat Ltd., Stockport, United Kingdom). The remaining 12 isolates (all *S. pneumoniae*) were provided as DNA extracts by Stephen Gillespie at The Royal Free Hospital, Hampstead, London, United Kingdom. MICs to penicillin had been determined for the original isolates by use of the agar dilution susceptibility test method (14).

Duplex real-time PCR. An 89-bp fragment of the *S. pneumoniae pbp2b* gene and a 101-bp fragment of the *lytA* gene were amplified in the following reaction mixture: 1× Quantitect multiplex master mix (Qiagen); 0.1 μM each of the primers LytA-F, LytA-R, Pbp-2b-F, and either Pbp-2b-R or Pbp-2b-RMOD (Table 1); 0.1 μM each of the probes LytA-probe and Pbp-2b-probe (Table 1); 5 μl of extracted DNA; and PCR-grade water up to a total volume of 40 μl. Reaction mixtures were cycled on the ABI Prism 7000 sequence detection system (Applied Biosystems, Warrington, United Kingdom) as follows: 50°C for 2 min, 95°C for 10 min, and then 45 cycles of 95°C for 15 s and 60°C for 1 min. The primer Pbp-2b-RMOD is a modified version of the published primer pbp-2b-R (8). Positive and negative controls were included in each run as described for the *pbp2b* PCR-RFLP method. Samples negative for both parts of the assay were subjected to broad-range 16S rRNA gene PCR, which includes a control for PCR inhibition (6).

Assay sensitivity. A wild-type, penicillin-susceptible (as determined by the BSAC disc sensitivity method [1]) *Streptococcus pneumoniae* isolate was used to determine the sensitivity of the real-time PCR assay. A suspension (with an optical density at 540 nm of 0.9) of the organism was prepared in 1 ml of sterile saline. The number of CFU in the suspension were estimated using a plate-counting method (12). DNA was extracted from the suspension by a mechanical lysis method as previously described (6). Serial 10-fold dilutions of extracted DNA were amplified in the PCR.

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Majority   ATTCTTGGTATACTCAGGCTTACGGTTCATTCCCTATCACAGCGGTCCAAGCTCTGGAGT
                    10        20        30        40        50        60

R6strain   ............................................................
S1         ............................................................
S2         ............................................................
S3         ............................................................
S4         ............................................................
S5         ............................................................
S6         ............................................................
I1         .....................A..C....A.........G..T......T..GG.G..CT..
I2         ..............GCCTT..CTC.GC..A.G..G..T..G....T...............
R1         ..........C...GCCTT...CT.TGC..A.G.....T..G..A..T..G.......A....
I3         ............AATT...A..T..A..T..T.......T......T..GG.....CT....
CS1        ............................................................
CS2        ...............................................A..............

Majority   ATTCATCAAATACCTATATGGTCCAAACA
                    70        80

R6 strain  .............................
S1         .............................
S2         .............................
S3         .......T.....................
S4         .......T.....................
S5         .......T.....................
S6         .......T.....................
I1         ....T..T...G.........T.....G..
I2         ...........G.T...............
R1         .......C...G.T...............
R2         .......C...G.T..C.....T.....C.
I3         ...........G.................
CS1        .............................
CS2        .......T.....................
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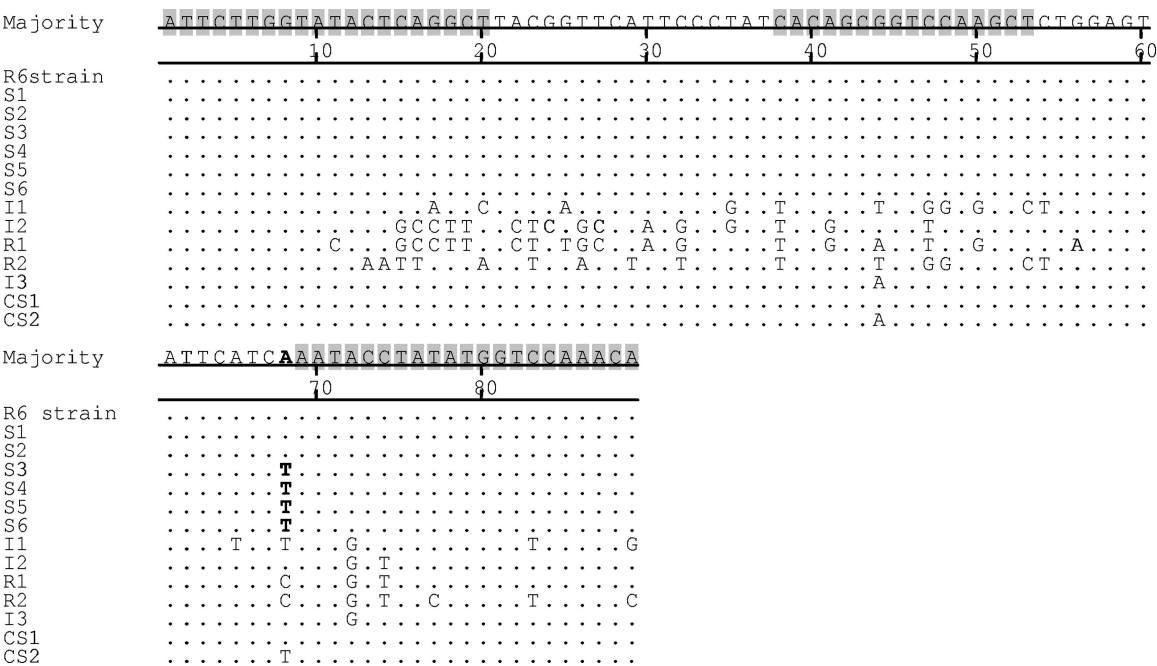
FIG. 1. Sequence alignment of an 89-bp region of the *pbp2b* gene from 10 *S. pneumoniae* isolates and the wild-type R6 strain (GenBank accession number AE008520). S indicates penicillin-susceptible strains (MICs of ≤0.06 mg/liter), I indicates strains with penicillin MICs of 0.12 of 1.0, and R indicates strains with penicillin MICs of >1.0 mg/liter. CS indicates *pbp2b* sequences derived from culture-negative clinical samples. The single-base polymorphism (A→T) at position 68 is located at the 3′ base of the original primer, Pbp-2b-R, is shown in bold type. Primer sequences are shaded gray.

pbp2b PCR-RFLP. The following is a modified version of a published method (2, 15). The PCR mixture was as follows: 1× Accuprime PCR buffer II (Invitrogen, Paisley, United Kingdom), 1 μl of Accuprime *Taq* polymerase (Invitrogen), 0.4 μM of each of the primers Pbp-2b-JT-F and Pbp-2b-JT-R (Table 1), 5 μl of extracted DNA, and PCR-grade UV-irradiated water to give a final volume of 50 μl. Reaction mixtures were heated to 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 60°C for 2 min, and 68°C for 3 min. A final extension was carried out at 68°C for 5 min. PCRs were electrophoresed through a 2% agarose gel containing 2 μl of 500 nM ethidium bromide at 100 V for 20 min, and bands were visualized by UV transillumination to confirm the presence of a 1,400-bp product. A positive control, DNA extracted from a penicillin-susceptible *S. pneumoniae* isolate, and a negative control, 5 μl of water, were included in each run.

PCR product (40 μl) was digested with HinfI (1 μl) in 1× buffer II (both from New England Biolabs, Hitchin, United Kingdom) at 37°C for 75 min. The entire digest was mixed with 4 μl of loading dye and run through a 2% agarose gel containing 2 μl of 500 nM ethidium bromide at 100 V for 3 h. Bands were visualized by UV transillumination and RFLP patterns compared to the pattern from the positive control (15).

Sequencing. The entire *pbp2b* gene was amplified as described above for the PCR-RFLP assay. A 273-bp fragment of the amplicon was sequenced with primers F2 and R4 and 89-bp sections of the resulting sequences were aligned with each other and the corresponding region from *S. pneumoniae* strain R6 (GenBank AE008520) by use of the CLUSTAL W algorithm in the MEGALIGN program (DNASTAR, Madison, WI). Details of the sequencing procedure have been described previously (6).

GenBank accession numbers. The sequences generated in this study have been deposited in GenBank under accession numbers EU274626 to EU274635 and EU293610 to EU293612.

RESULTS

Assay validation. (i) Sensitivity. The duplex PCR was applied to a dilution series of DNA prepared from a quantified suspension of *S. pneumoniae*. The detection limit for both the *lytA* and *pbp2b* targets was 0.5 CFU per reaction. This is around 100-fold more sensitive than our broad-range 16S rRNA gene PCR (6).

DNA extracts from 12 penicillin-susceptible *S. pneumoniae* isolates were amplified in the duplex assay with the published reverse primer Pbp-2b-R (Table 1). All 12 strains were *lytA* PCR and *pbp2b* PCR positive; in four cases, however, the *pbp2b* product was detected around 10 cycles later than that in the *lytA* PCR, which is equivalent to a reduction in PCR sensitivity of around 1,000-fold. This observation could be explained by mismatches in the primer and probe sequences that have reduced PCR efficiency. To investigate this, the entire *pbp2b* gene was amplified for these four isolates (S3 to S6), two other susceptible isolates (S1 and S2), two isolates with MICs of >1.0 mg/liter (R1 and R2), and two isolates with MICs of 0.12 to 1.0 mg/liter (I1 and I2). A 273-bp fragment of the resulting amplicons was sequenced in both directions. Figure 1 shows the sequence alignment of the 89-bp sequence corresponding to the real-time PCR product. The four isolates with MICs of >0.6 have several mutations compared to the wild-type R6 sequence. All four of the susceptible isolates that were *pbp2b* PCR positive, but with a much larger cycle threshold (C_T) value than the *lytA* PCR run simultaneously (S3 to S6), have a single-nucleotide polymorphism (A→T) at position 68 compared to the wild-type sequence from strain R6 and susceptible isolates S1 and S2. This is the 3′-terminal base of the primer Pbp-2b-R, and mismatched primer binding would explain the reduced efficiency of the *pbp2b* PCR for these four isolates. We modified the primer by shifting it downstream by one nucleotide. This modification does not affect the melting temperature or specificity of the primer and allows efficient
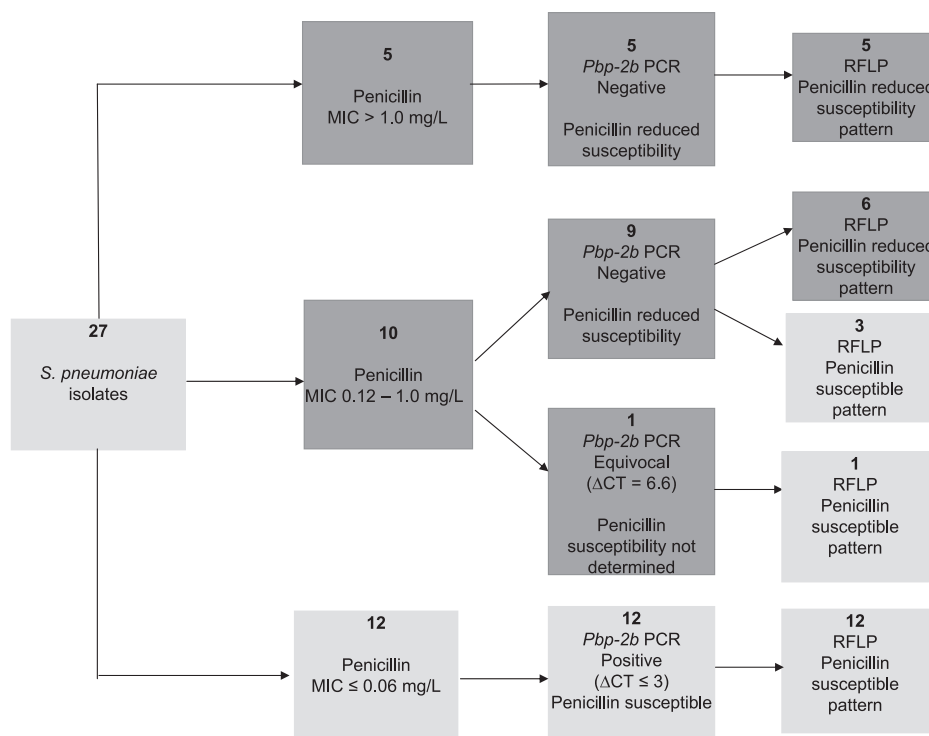
FIG. 2. Flow chart showing the relationship between drug MICs for 27 *S. pneumoniae* isolates, *pbp2b* PCR results, and PCR-RFLP patterns. An equivocal *pbp2b* PCR result is positive with a C_T value of more than 3 cycles greater than the *lytA* C_T value ($\Delta C_T > 3$) when the assays were run simultaneously.

amplification of all 12 penicillin-susceptible isolates. The modified primer, Pbp-2b-RMOD (Table 1), was used for the rest of the study.

(ii) Specificity. (a) *lytA* PCR. *lytA* PCR, as part of the duplex assay, was performed on 35 isolates, i.e., 27 *S. pneumoniae* and 8 SMG isolates. This assay can be positive (*S. pneumoniae* detected) or negative (*S. pneumoniae* not detected). All 27 *S. pneumoniae* isolates and 3 SMG isolates were *lytA* PCR positive. Forty-six DNA extracts from clinical samples previously positive for *S. pneumoniae* by 16S rRNA gene PCR (but not culture) were also tested in the duplex assay, and all of them were positive by *lytA* PCR.

(b) *pbp2b* real-time PCR. The 27 *S. pneumoniae* isolates described above were subjected to a *pbp2b* real-time PCR (as part of the duplex assay). For the five isolates with penicillin MICs of >1.0 mg/liter, the *pbp2b* PCR was negative. For the 10 isolates with penicillin MICs of 0.12 to 1.0 mg/liter, the *pbp2b* PCR was negative for 9 and positive for 1 (the latter result was later reclassified as equivocal; see below). For the 12 penicillin-susceptible isolates (MICs of ≤0.06 mg/liter), the *pbp2b* PCR results were positive. The difference between the C_T values (ΔC_T) for the *pbp2b* PCR and the *lytA* PCR that was run simultaneously was calculated for each of the 13 isolates that were *pbp2b* PCR positive (data not shown). All 12 penicillin-susceptible isolates had a ΔC_T value of 3 cycles or less, and the isolate with a penicillin MIC of 0.12 to 1.0 had a ΔC_T value of 6.6. Based on these results, we devised the following simple algorithm for the interpretation of *pbp2b* real-time PCR results: a penicillin-susceptible result is *pbp2b* PCR positive with a C_T value of no more than 3 cycles greater ($\Delta C_T \leq 3$) than

that for the *lytA* PCR run simultaneously, while a reduced penicillin susceptibility result is *pbp2b* PCR negative. Penicillin susceptibility is not determined for equivocal *pbp2b* PCR results (positive, but with a C_T value of more than 3 cycles greater [$\Delta C_T > 3$] than that for the *lytA* PCR run simultaneously) (Fig. 2).

Forty-six archived DNA extracts from clinical samples, previously positive for *S. pneumoniae* by 16S rRNA gene PCR, were tested in the duplex assay. Forty-one samples were *pbp2b* PCR positive and 5 were negative. The above algorithm was used to interpret the results as follows: 39 samples were penicillin susceptible and 5 had reduced penicillin susceptibility. Penicillin susceptibility was not determined for two samples with equivocal *pbp2b* PCR results (ΔC_T values of 3.5 and 5.7) (Fig. 3).

(c) *pbp2b* PCR-RFLP. The same 27 *S. pneumoniae* isolates were subjected to *pbp2b* PCR-RFLP. This assay can give the penicillin-susceptible RFLP pattern or one of several reduced penicillin susceptibility patterns. The five isolates with penicillin MICs of >1.0 mg/liter all gave a reduced penicillin susceptibility pattern. Of the 10 isolates with penicillin MICs of 0.12 to 1.0 mg/liter, 6 gave a reduced penicillin susceptibility pattern and 4 gave the penicillin-susceptible pattern. All 12 of the penicillin-susceptible isolates (MICs of ≤0.06 mg/liter) gave the penicillin-susceptible RFLP pattern (Fig. 2).

The 46 DNA extracts were also subjected to *pbp2b* PCR-RFLP, and all 39 samples that were penicillin susceptible by *pbp2b* PCR and the 2 samples with equivocal *pbp2b* PCR results gave the penicillin-susceptible RFLP pattern. Of the five samples that were classified as having reduced penicillin
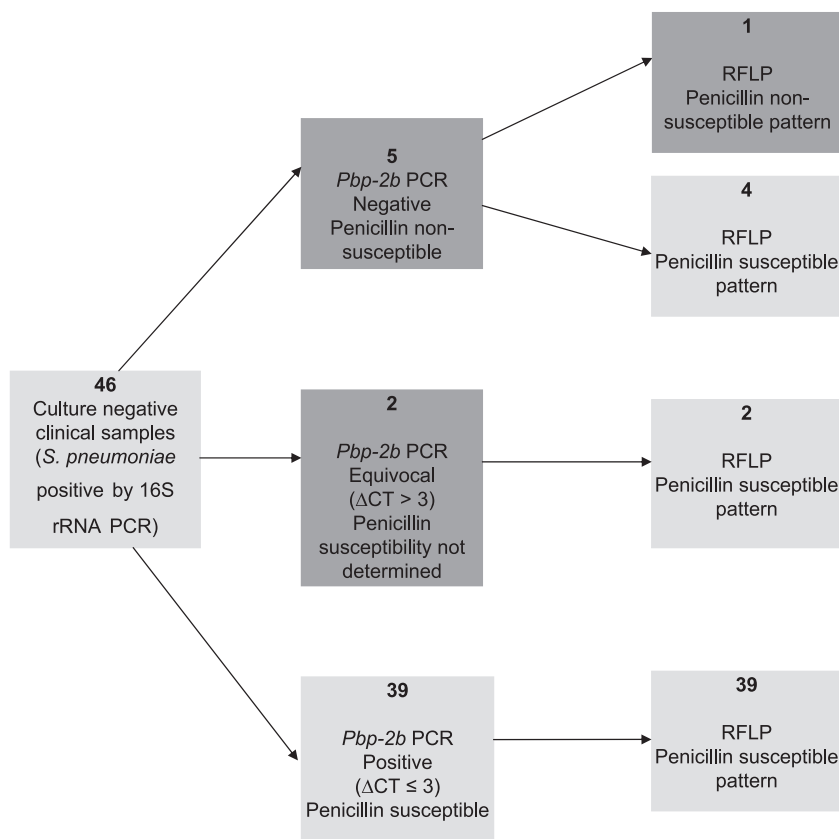
FIG. 3. Flow chart showing how penicillin susceptibility would be assigned by real-time *pbp2b* PCR (or PCR-RFLP) in 46 cases of culture-negative *S. pneumoniae* infection. An equivocal *pbp2b* PCR result is positive with a C_T value of more than 3 cycles greater than the *lytA* C_T value ($\Delta C_T > 3$) when the assays are run simultaneously.

susceptibility by *pbp2b* PCR, only one gave a reduced penicillin susceptibility RFLP pattern, while the remaining four samples gave the penicillin-susceptible pattern (Fig. 3).

The validation of the assay has demonstrated that the PCR-RFLP assay could not reliably detect penicillin MICs of 0.12 to 1.0 mg/liter, and therefore it was not applied in prospective analysis of clinical samples.

(d) *pbp2b* sequence data. A 273-bp fragment of the *pbp2b* gene was sequenced for one isolate (I3) that had a penicillin MIC of 0.12 to 1.0 mg/liter and an equivocal *pbp2b* real-time PCR result. This revealed two mutations in the 89-bp region of interest, one of which was within the probe site (Fig. 1).

Sequencing of the *pbp2b* gene was also performed for two DNA extracts from clinical samples that gave equivocal real-time PCR results. One of these samples (CS1) had the wild-type *pbp2b* sequence ($\Delta C_T = 3.5$), and the other (CS2) had two mutations within the primer and probe binding sites ($\Delta C_T = 5.7$) (Fig. 1).

Prospective routine clinical application of the assay. Two hundred culture-negative clinical specimens sent to the laboratory for broad-range 16S rRNA gene PCR were also tested as part of the routine diagnostic algorithm in the duplex real-time *S. pneumoniae* PCR if *S. pneumoniae* infection was suspected from the clinical details. One hundred six of these samples were *lytA* PCR negative and *S. pneumoniae* DNA negative by broad-range 16S rRNA gene PCR (22 samples

were positive for another organism). Ninety-four specimens (from 70 patients) were *lytA* PCR positive, 37 of these were 16S rRNA gene PCR negative (or in two cases positive for another organism), 17 samples did not have a 16S rRNA gene PCR performed (because the *lytA* PCR was strongly positive), and 40 samples were also 16S rRNA gene PCR positive. Of these 40 samples, 13 were confirmed as *S. pneumoniae* positive by 16S rRNA gene sequencing, while the remaining 27 samples were presumed to be *S. pneumoniae* positive by 16S rRNA gene PCR because of the strongly positive *lytA* PCR result.

Of the 94 *lytA* PCR-positive samples, 70 samples (from 57 patients) were also *pbp2b* PCR positive, with C_T values within 3 cycles of the *lytA* PCR run simultaneously ($\Delta C_T \leq 3$), and therefore were interpreted as penicillin susceptible. Nine samples (from eight patients) gave equivocal *pbp2b* PCR results, that is, results that were positive but with a ΔC_T value of greater than 3; penicillin susceptibility was not determined. Fifteen *lytA* PCR-positive samples (from 11 patients) were *pbp2b* PCR negative, and 14 of these were interpreted as having reduced penicillin susceptibility. The remaining sample was *lytA* PCR positive, with a C_T value of 40; therefore, susceptibility to penicillin could not be determined due to the unreliable performance of PCR after so many cycles (Fig. 4).

Twenty-four patients had more than one sample that was *lytA* PCR positive. For 19 of these patients, the interpretation of penicillin susceptibility based on the *pbp2b* PCR result was
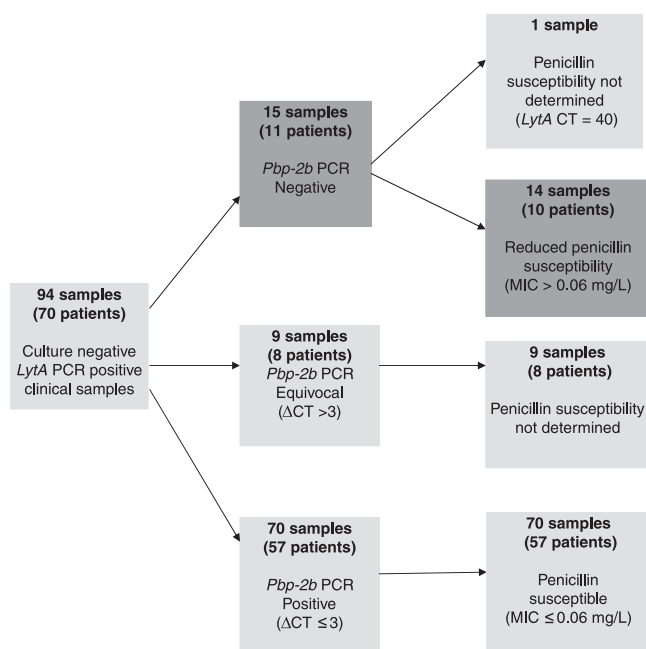
FIG. 4. Flow chart showing the interpretation of penicillin suscep-
tibility for 94 clinical specimens (from 70 patients), all *S. pneumoniae*
positive (by *lytA* PCR). A positive real-time PCR result implies sus-
ceptibility to penicillin (MICs of ≤0.06 mg/liter) and a negative result
implies reduced penicillin susceptibility (MICs of >0.06 mg/liter). Pen-
icillin susceptibility was not determined for equivocal *pbp2b* PCR re-
sults, that is, results that were positive with a C_T value of more than 3
cycles greater than the *lytA* C_T value ($\Delta C_T > 3$) when the assays were
run simultaneously.

the same for all samples. The remaining five patients (with
between two and seven samples each) had at least one discrep-
ant result. Two patients each had one sample interpreted as
penicillin susceptible and another as equivocal; one patient
had two penicillin-susceptible samples and one equivocal sam-
ple; one patient had two penicillin-susceptible samples, one
equivocal sample, and one sample for which penicillin suscep-
tibility could not be determined; and one patient had five
reduced-penicillin-susceptibility samples and two samples that
were equivocal.

DISCUSSION

The aim of this study was to create a sensitive duplex PCR
assay that can simultaneously detect *lytA* and *pbp2b* gene tar-
gets in a single real-time PCR, providing rapid diagnosis of
Streptococcus pneumoniae infection, along with penicillin sus-
ceptibility data, for culture-negative clinical specimens. Initial
validation of a previously published real-time *pbp2b* PCR (8) in
our laboratory revealed that it does not amplify all penicillin-
susceptible *S. pneumoniae* strains with equal efficiencies, due to
a single-nucleotide mismatch at the 3′ end of the reverse
primer. Modification of the primer resulted in a sensitive PCR
for the detection of all penicillin-susceptible strains.

The turn-around time for the assay, including extraction
of DNA from the sample, is around 3 hours. This is faster
than any conventional PCR and significantly faster than
broad-range 16S rRNA gene PCR, which requires sequenc-

ing of PCR products. With additional PCR-RFLP analysis
of the *pbp2b* gene, the entire procedure takes around 72 h
(2, 6, 15).

During assay validation, all 27 *S. pneumoniae* isolates tested
in the assay and 46 clinical samples tested that were *S. pneu-
moniae* positive by broad-range 16S rRNA gene PCR were *lytA*
PCR positive. This demonstrates the ability of the assay to
detect *S. pneumoniae* DNA. It has been reported in the liter-
ature that strains of closely related oral streptococci, in partic-
ular the SMG strains, may also harbor the *lytA* gene in addition
to other *S. pneumoniae*-specific virulence factors such as pneu-
molysin (20, 21). These atypical isolates often display unusual
phenotypic characteristics, such as optochin sensitivity, that
make them appear more like *S. pneumoniae* isolates. It must be
noted, though, that these "atypical" oral streptococci are often
highly virulent and isolated from patients with significant pneu-
mococcal infection-like clinical conditions (21). In this study,
we tested eight SMG isolates, all of which were causing clini-
cally significant infections, were felt to be "atypical" or "*S.
pneumoniae*-like," and had required 16S rRNA gene sequenc-
ing for identification. Three of these isolates were *lytA* PCR
positive. These data support the idea that the *lytA* gene may
not be specific for *S. pneumoniae*. However, if the *lytA* PCR
gives false-positive results for SMG organisms that are causing
serious clinical infections, we would argue that this "nonspeci-
ficity" does not render the assay any less useful from a clinical
perspective.

During assay validation, five isolates with penicillin MICs of
>1.0 mg/liter and 10 isolates with penicillin MICs of 0.12 to 1.0
mg/liter were tested. Fourteen of these isolates were *pbp2b*
real-time PCR negative, and one isolate (MIC of 0.12 to 1.0
mg/liter) gave an equivocal result ($\Delta C_T = 6.6$) (Fig. 2). Se-
quencing of the *pbp2b* gene for the equivocal isolate (I3) re-
vealed two mutations compared to the wild-type sequence in
the region analyzed (Fig. 1). One of these was within the probe
sequence and would have affected probe binding. None of
these 15 isolates with penicillin MICs of >0.06 mg/liter would
have been classified as penicillin susceptible by the duplex
assay (Fig. 2). During assay validation, 46 DNA extracts were
tested. Thirty-nine samples were *pbp2b* real-time PCR positive
and can be interpreted as penicillin susceptible. Five samples
were *pbp2b* real-time PCR negative and would be interpreted
as having reduced penicillin susceptibility, while two samples
gave an equivocal *pbp2b* real-time PCR result, and no inter-
pretation of penicillin susceptibility is possible (Fig. 3). How-
ever, one of these equivocal samples (CS1) had a ΔC_T value of
close to 3, and it is likely that this sample contains DNA from
a fully sensitive organism. The other equivocal sample (CS2)
had a ΔC_T value of greater than 5 and is therefore was more
likely to derive from an isolate with a penicillin MIC of >0.06
mg/liter. The sequence data for these two samples also support
this, with the first isolate ($\Delta C_T = 3.5$) having a sequence iden-
tical to that of the wild-type strain in the region of the *pbp2b*
gene analyzed. The other isolate ($\Delta C_T = 5.7$) had two muta-
tions in the region of the *pbp2b* gene analyzed, one of which
would have affected probe binding (Fig. 1).

PCR-RFLP analysis did not detect all of the isolates with
penicillin MICs of 0.12 to 1.0 mg/liter; four were classified as
penicillin susceptible. Additionally, four of the five DNA ex-
tracts that were determined to indicate reduced penicillin sus-

ceptibility by real-time *pbp2b* PCR gave the penicillin-susceptible RFLP pattern. We concluded that PCR-RFLP is not a reliable method for determining penicillin susceptibility (MICs of ≤0.06 mg/liter). However, this validation has demonstrated that the real-time *pbp2b* PCR can accurately differentiate between penicillin-susceptible isolates (MICs of ≤0.06 mg/liter) and isolates with reduced susceptibility to penicillin (MICs of >0.06 mg/liter) and could provide clinically useful data on penicillin susceptibility in *S. pneumoniae* PCR-positive, culture-negative clinical specimens.

The assay was applied prospectively to 200 culture-negative clinical samples sent to the laboratory for routine analysis by broad-range 16S rRNA gene PCR. Ninety-four samples (from 70 patients) were *lytA* PCR positive. Thirty-seven of these samples were broad-range PCR negative (or in two cases positive for another organism), demonstrating an improvement in the diagnosis of *S. pneumoniae* in culture-negative specimens due to the increased sensitivity of the real-time PCR compared to that of the broad-range assay.

Seventy of the *lytA* PCR-positive samples (from 57 patients) were also *pbp2b* PCR positive ($\Delta C_T \leq 3$). The validation of the assay allows us to be confident in our interpretation of these 70 samples as positive for penicillin-susceptible *S. pneumoniae* (MICs of ≤0.06 mg/liter). Fifteen *lytA* PCR-positive samples (from 11 patients) were *pbp2b* PCR negative, and 14 of these were interpreted as having reduced penicillin susceptibility (MICs of >0.06 mg/liter). The remaining sample was very weakly positive in the *lytA* PCR ($C_T = 40$). The *pbp2b* PCR result for this sample was negative but penicillin susceptibility could not be determined because amplification of the *pbp2b* target may not be reliable in the last few cycles of a PCR assay.

The remaining nine *lytA* PCR-positive samples (from eight patients) gave equivocal *pbp2b* PCR results (that is, positive with a ΔC_T value of >3). Three of these samples (from two patients) had very large ΔC_T values (greater than 7) and could be considered to have probable reduced susceptibility to penicillin based on the observation during the validation of the assay that one penicillin isolate with an MIC of 0.12 to 1.0 mg/liter gave an equivocal *pbp2b* PCR result, with a ΔC_T value of 6.6.

Interestingly, four of the nine *pbp2b* PCR-equivocal samples (ΔC_T values of between 3 and 5) were from patients who also had at least one other sample interpreted as penicillin susceptible. A further two *pbp2b* PCR-equivocal samples (ΔC_T values of greater than 7) were from a patient who had five other samples that were *pbp2b* PCR negative and therefore were interpreted as indicating reduced penicillin susceptibility.

Two samples that were positive for reduced-penicillin-susceptibility *S. pneumoniae* were from two patients that subsequently yielded *S. pneumoniae*, reported as penicillin susceptible, upon culture from specimens taken at their referring hospitals. Review of susceptibility testing in the referring hospital showed that one isolate had a penicillin MIC of 0.12 to 1.0 mg/liter by Vitek (Biomerieux UK Ltd., Basingstoke, United Kingdom) and was penicillin susceptible by the BSAC disc sensitivity method (1). The other isolate was classified as having reduced penicillin susceptibility (MIC of >0.06 mg/liter), but a transcriptional error had occurred during reporting.

This study has shown that a duplex PCR for *lytA* and *pbp2b* gene targets can be applied to culture-negative samples to improve the diagnosis of *S. pneumoniae* infection. The assay can confidently deduce penicillin susceptibility (MICs of ≤0.06 mg/liter) in the majority (89%) of *S. pneumoniae* PCR-positive samples; the assay is fast and reliable enough to use to facilitate the rational reduction of antibiotic spectrum for the appropriate treatment of *S. pneumoniae* infection.

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